Purification and Characterization of δ Helicase from Fetal Calf Thymus†

Xiangyang Li,‡,§ Cheng-Keat Tan,‖ Antero G. So,‡,‖ and Kathleen M. Downey*,‡,‖
Departments of Medicine and Biochemistry/Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101
Received September 23, 1991; Revised Manuscript Received January 23, 1992

ABSTRACT: A DNA helicase (δ helicase) which partially copurifies with DNA polymerase δ has been highly purified from fetal calf thymus. δ helicase differs in physical and enzymatic properties from other eukaryotic DNA helicases described thus far. The enzyme has an apparent mass of 57 kDa by gel filtration and is associated with polypeptides of 56 and 52 kDa by SDS–polyacrylamide gel electrophoresis. Photo-cross-linking of the purified enzyme with [α-^{32}P]ATP resulted in labeling of a polypeptide of approximately 58 kDa, suggesting that the active site is present on the larger polypeptide. Unwinding of a partial duplex requires a nucleoside triphosphate which can be either ATP or dATP but not a nonhydrolyzable analogue of ATP. Other ribo- and deoxyribonucleoside triphosphates have little or no activity as cofactors. δ helicase also has DNA-dependent ATPase activity which has a relatively low K_m for ATP (40 μM). δ helicase binds to single-stranded DNA but has little or no affinity for double-stranded DNA or single-stranded RNA. Similar to replicative DNA helicases from prokaryotes and the herpes simplex virus type 1 helicase–primase, δ helicase translocates in the 5′–3′ direction along the strand to which it is bound and preferentially unwinds DNA substrates with a forklike structure.

Recent studies suggest that the mechanism of DNA replication is similar in both prokaryotes and eukaryotes; i.e., the concerted action of multiple proteins is required for replication fork movement (McHenry, 1988; Alberts, 1990; Kornberg & Baker, 1991). DNA helicases which function to unwind the parental DNA strands are essential components of the replication machinery, both to unwind replication origins during the initiation phase of DNA replication and to separate parental DNA strands during the elongation phase, thereby creating single-stranded templates for both leading- and lagging-strand synthesis. The enzymatic unwinding of duplex DNA by a DNA helicase, fueled by the hydrolysis of a nucleoside 5′-triphosphate, involves the unidirectional translocation of the helicase along one DNA strand, thus displacing the other strand (Matson & Kaiser-Rogers, 1990).

Several DNA helicases responsible for fork movement during the replication of bacterial and phage genomes have been purified and characterized. These helicases, e.g., *Escherichia coli dnaB* helicase (LeBowitz & McMacken, 1986), T4 gene 41 helicase (Richardson & Nossel, 1989), and T7 gene 4 helicase (Matson et al., 1983), have several properties in common: they all translocate in the 5′ to 3′ direction along the lagging-strand template, they all either require or prefer a replication fork-like DNA substrate, and they are all tightly associated with a DNA primase.

Our knowledge of the helicases involved in the replication of eukaryotic genomes is considerably less (Matson & Kaiser-Rogers, 1990; Thommes & Hubscher, 1990a). In recent years DNA helicases have been isolated from a number of eukaryotic sources including animal viruses (Stahl et al., 1986; Wiekowski et al., 1988; Goetz et al., 1988; Crute et al., 1988; Bruckner et al., 1991), calf thymus tissue (Thommes & Hubscher, 1990b), mouse cells (Seki et al., 1987, 1988), human cells (Daily et al., 1990; Tuteja et al., 1991; Seo et al., 1991), *Xenopus laevis* ovaries (Poll & Benbow, 1988), and yeast (Sugino et al., 1986; Sung et al., 1987; Harosh et al., 1989). Several viral DNA helicases have been highly purified and well characterized. Simian virus 40 (SV40) large T antigen, which has both origin-binding and DNA helicase activities, is the only virally encoded protein required for SV40 DNA replication (Stahl & Knippers, 1987; Kelly, 1988; Stillman, 1989). The helicase activity of T antigen is required for initiation of replication at the SV40 origin and is also thought to function during the elongation phase of replication to open the parental helix (Stahl et al., 1985, 1986). Unlike the prokaryotic replicative DNA helicases, T antigen has been shown to translocate in the 3′ to 5′ direction, presumably along the leading-strand template (Wiekowski et al., 1988). The herpes simplex virus type 1 (HSV-1) origin binding protein, the product of the UL9 gene, also has helicase activity, although the direction of translocation has not been determined (Bruckner et al., 1991). However, the HSV-1 helicase–primase, the product of the UL52, UL5, and UL8 genes, unwinds duplex DNA by translocating in the 5′ to 3′ direction along the lagging-strand template, similar to the prokaryotic replicative DNA helicases (Crute et al., 1988, 1989; Crute & Lehman, 1991; Dodson & Lehman, 1991). Several cellular DNA helicases have also been purified and studied; however, their in vivo roles are less clear and it has not been ascertained whether any of these DNA helicases are involved in replication of genomic DNA.

Here we present data on the purification and characterization of a DNA helicase from fetal calf thymus which shares several properties with replicative DNA helicases. Because it separates from calf thymus DNA polymerase δ (pol δ) in the final step of purification of the polymerase, as well as to distinguish it from a recently described DNA helicase from calf thymus which has very different properties (Thommes & Hubscher, 1990b), we have called this calf thymus DNA helicase δ helicase.

Materials and Methods

Materials. Poly(dT), poly(A), deoxyribonucleoside triphosphates (dNTPs), ribonucleoside triphosphates (NTPs), ADP, AMP, terminal deoxynucleotidyltransferase, pBR322 DNA, the Klenow fragment of *E. coli* DNA polymerase I, and

† This work was supported by National Institutes of Health Grant DK26206.
* To whom correspondence should be addressed at the Department of Medicine (R-99), University of Miami, P.O. Box 016960, Miami, FL 33101.
‡ Department of Biochemistry/Molecular Biology.
§ Present address: Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis, MO 63110.
‖ Department of Medicine.

0006-2960/92/0431-3507$03.00/0

FPLC columns were from Pharmacia-LKB Biotechnology. Poly(dA-dT), poly(dA), and $(dT)_{12-15}$ were from Midland Certified Reagents. [^{3}H]dTTP, [α-^{32}P]dNTPs, [α-^{32}P]ATP, and [γ-^{32}P]ATP were from ICN. The ATP analogues AMP-PNP,[1] AMP-PCP, and ATP-γ-S and molecular weight standards for gel filtration were from Boehringer Mannheim Biochemicals. T4 polynucleotide kinase was from U.S. Biochemicals. M13mp19 (+)-strand DNA, the 17-base M13/pUC forward sequencing primer, heparin–agarose, and single-stranded DNA–agarose were from BRL. PEI-Cellulose sheets were from Brinkman Instruments, Inc. Protein molecular weight markers and prestained protein molecular weight markers were from Bio-Rad. Fetal calf thymus was from Antech Inc. Spun columns were from 5′ to 3′ Inc.

Oligonucleotides for preparation of helicase substrates were synthesized by Midland Certified Reagent Co. The sequences of the oligonucleotides are as follows: (A) 5′-CCC ATT GCG GTC CCA AAA GGG TCA GTG GAC GTT GTA AAA CGA CGG CCA GT-3′; (B) 5′-GAC GTT GTA AAA CGA CGG CCA GTC TTA AGC TCG AGC CAT GGG CCC CTA GG-3′; (C) 5′-AGT CAC GAC GTT GTA-3′; (D) 5′-TCT CTC TCT CTC TCT CTC TCT CTC TCT CTC TTT TTT ACA ACG TCG TGA CT-3′; and (E) 5′-TTT TTT ACA ACG TCG TGA CTC TCT CTC TCT CTC TCT CTC TCT CTC TCT CT-3′.

Proteins. DNA polymerase δ was purified from fetal calf thymus through step 6 as described previously (Lee et al., 1984). The proliferating cell nuclear antigen (PCNA) was purified from fetal calf thymus as described (Tan et al., 1986).

Polynucleotides. [^{3}H]-Labeled $(dT)_{50}$ (260 cpm/pmol of dTMP) was synthesized with terminal deoxynucleotidyltransferase as previously described (Que et al., 1978). The helicase substrates used for measuring direction of translocation were prepared according to Weikowski et al. (1988). The 15-mer C was annealed to the 50-mers D and E at a molar ratio of 1:3 and 3′-labeled with [^{32}P] by incubation with 5 units of the Klenow fragment of *E. coli* DNA polymerase I and 0.1 μM [α-^{32}P]dATP (8×10^8 cpm/pmol). After 20 min at 25 °C, 25 μM dATP was added and the mixture was further incubated for 20 min at 25 °C. Unincorporated nucleotides were removed by centrifugation of the partial duplex through a Select-D, G-25 column. The structures of these helicase substrates are shown in Figure 7.

Forklike helicase substrates were prepared as follows. The 50-mers A and B were labeled with [^{32}P] at their 5′ termini by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase (Maniatis et al., 1982). The unreacted [γ-^{32}P]ATP was removed by centrifugation of the labeled oligonucleotide through Select-D, G25. The labeled 50-mers were annealed to M13mp19 (+)-strand DNA at a molar ratio of 3:1, and unannealed 50-mer was removed by centrifugation through Select-5L. The 3′-end of 50-mer A is complementary to nucleotides 6293–6313 of M13mp19 (+)-strand DNA and produced a forklike substrate with a 5′-tail (50-mer, 5′-tailed). The 5′-end of 50-mer B is complementary to nucleotides 6293–6313 of M13mp19 (+)-strand DNA and produced a forklike substrate with a 3′-tail (50-mer, 3′-tailed). An untailed substrate was prepared by annealing the 17-base forward sequencing primer pUC/M13 to M13mp19 (+)-strand DNA at a molar ratio of 4:1 and 3′-labeled by incubation with 15 units of Klenow fragment, 1 μM [α-^{32}P]dATP, 1 μM [α-^{32}P]dTTP, and 100 μM dGTP for 20 min at 25 °C, followed by the addition of 30 μM dATP and 30 μM dTTP and further incubation at 25 °C for 20 min. The partial duplex was separated from unincorporated nucleotides and unannealed oligomer by centrifugation through Select-5L. The untalied substrate is complementary to nucleotides 6286–6307 of M13mp19 (+)-strand DNA (22-mer, untailed). The structures of these helicase substrates are depicted schematically in Figure 8.

DNA Polymerase Assay. DNA polymerase δ was assayed as previously described (Tan et al., 1986). The reaction mixture contained, in a final volume of 60 μL, 40 mM Bis-Tris buffer, pH 6.5, 6 mM $MgCl_2$, 40 μg/mL BSA, 1 mM DTT, 10% glycerol, 15 μg/mL poly(dA)·oligo(dT) (20:1), 40 μM [^{3}H]dTTP (200 cpm/pmol), and 4 μg/mL PCNA. After 15 min at 37 °C the samples were precipitated with TCA, collected on GF/C filters, washed, and counted as described previously (Tan et al., 1986).

3′–5′ Exonuclease Assay. 3′–5′ exonuclease activity was assayed as previously described (Lee et al., 1984) with [^{3}H]-labeled $(dT)_{50}$ (260 cpm/pmol of dTMP) as substrate. The final reaction volume was 50 μL, and incubation was for 15 min at 37 °C. [^{3}H]-Labeled polynucleotide remaining was quantitated by spotting aliquots of reaction mixtures on Whatman DE-81 filters and washing with 0.3 M ammonium formate.

DNA-Dependent ATPase Assay. ATPase activity was assayed essentially as described by Matson and Richardson (1983). The reaction mixture contained, in a final volume of 25 μL, 40 mM Bis-Tris buffer, pH 6.5, 2 mM $MgCl_2$, 40 μg/mL BSA, 4 mM DTT, 5 nmol of [α-^{32}P]ATP or [α-^{32}P]dATP (100 cpm/pmol) and 90 μM poly(dT). After 30 min at 37 °C, 10 μL of the reaction mixture was spotted on a PEI-cellulose plate, prespotted with 33 nmol each of ATP, ADP, and AMP. After drying, the plate was developed in 1 M formic acid for 2 cm and then in 1 M LiCl–1 M formic acid for 15 cm. ADP, located by UV light, was excised and counted.

Helicase Assay. The reaction mixture contained, in a final volume of 25 μL, 80 mM sodium phosphate buffer, pH 6.5, 100 μg/mL BSA, 3 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, and 3–10 ng of labeled DNA substrate. After incubation at 37 °C for the indicated time, the reaction was stopped by the addition of 0.3 volume of a mixture of 1% SDS, 33 mM EDTA, and 33% glycerol. The mixture was electrophoresed through a nondenaturing 8 or 12% polyacrylamide gel in Tris–borate–EDTA, pH 8.0, at 25 °C. The products were visualized by autoradiography.

Helicase activity was quantitated either by excising radioactive bands corresponding to the substrates and displaced fragments and counting or by scanning X-ray films with a laser densitometer. The quantity of fragment displaced was estimated from the fraction of the total recovered [^{32}P] signal in the free fragment band, as described by Oh and Grossman (1989). One unit of helicase is defined as the displacement of 15% of the substrate in 30 min at 37 °C with a forklike substrate (50-mer, 3′-tailed).

SDS–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed according to Laemmli (1970) and proteins were detected by silver staining according to Wray et al. (1981).

Photo-Cross-Linking. Photo-cross-linking of helicase protein with [α-^{32}P]ATP was performed essentially as described

[1] Abbreviations: AMP-PCP, 5′-adenylyl methylenediphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PAGE, polyacrylamide gel electrophoresis.

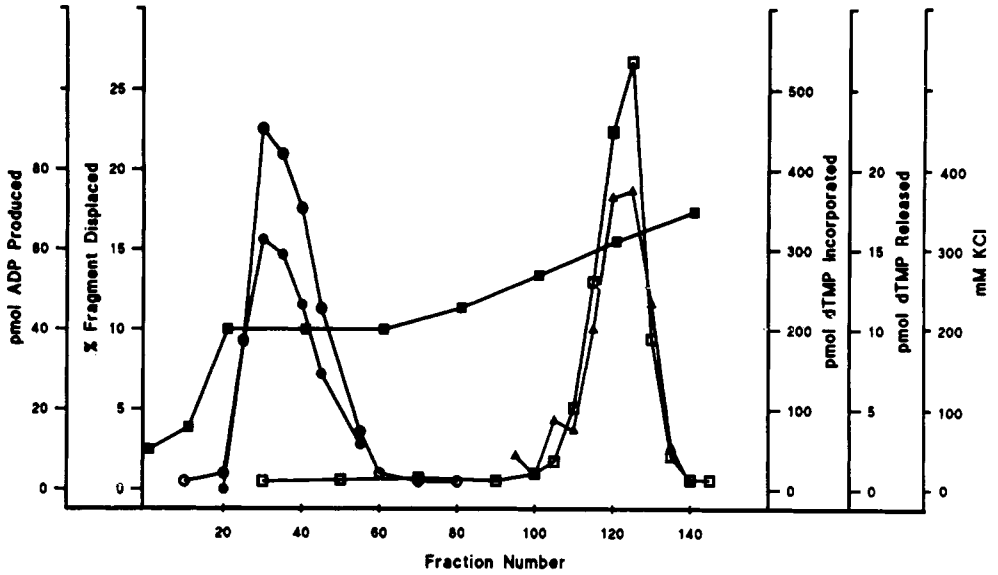

FIGURE 1: Separation of DNA helicase and DNA-dependent ATPase from DNA polymerase δ on heparin–agarose chromatography. Experimental details are given in the text. Enzyme assays are as described in Materials and Methods. DNA helicase activity (○) was assayed with the partial duplex containing a 3′-tail (50-mer, 3′-tailed) and incubation was for 60 min. ATPase activity (●), DNA polymerase activity (□), 3′–5′ exonuclease activity (▲), and KCl concentration (■) are shown.

(Biswas & Kornberg, 1984; Foiani et al., 1989). Protein was mixed with [α-^{32}P]ATP and the mixture was illuminated with a 254-nm UV light for 10 min. The proteins were precipitated with 20% TCA, dissolved in a buffer of 62.5 mM Tris-HCl, pH 6.8, 12% glycerol, 0.002% bromophenol blue, 2% SDS, and 5% 2-mercaptoethanol, heated at 95 °C for 10 min, and electrophoresed on an SDS–10% polyacrylamide gel. The radioactive polypeptide was located by autoradiography.

RESULTS

Purification of δ Helicase. The starting material for the purification of δ helicase was partially purified (step 6) DNA polymerase δ from fetal calf thymus (Lee et al., 1984). Figure 1 shows the separation of DNA helicase and DNA-dependent ATPase activities from DNA polymerase δ on heparin–agarose chromatography. Approximately 39 000 units of pol δ [step 6 according to Lee et al. (1984)] were loaded on a 2.2 × 5.5 cm heparin–agarose column in TGEEDP buffer (50 mM Tris-HCl, pH 7.8, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2 mM PMSF) containing 50 mM KCl. Stepwise elution of the column with TGEEDP buffer containing 0.2 M KCl resulted in the elution of DNA helicase and DNA-dependent ATPase activities, while DNA polymerase δ core enzyme was subsequently eluted at approximately 0.3 M KCl. Since the 3′–5′ exonuclease activity of pol δ degrades the helicase product and/or substrate, it was not possible to assay helicase activity until it had been separated from DNA polymerase δ. Consequently it was not possible to determine whether the DNA helicase truly copurifies with pol δ during early purification steps or whether it is fortuitously present in step 6 pol δ.

Further purification of δ helicase on single-stranded DNA–agarose chromatography is shown in Figure 2. Approximately 26 000 units of helicase (8200 units/mg) were loaded on a 6.6-mL single-stranded DNA–agarose column in BTGEEDP buffer (the same as TGEEDP buffer except that 50 mM Tris-HCl, pH 7.8, is replaced by 50 mM Bis-Tris-HCl, pH 6.5). Stepwise elution with 150 mM KCl in BTGEEDP buffer resulted in the elution of a large protein peak but little or no helicase activity, while a 400 mM KCl step resulted in the elution of the helicase activity. Approximately 50% of the

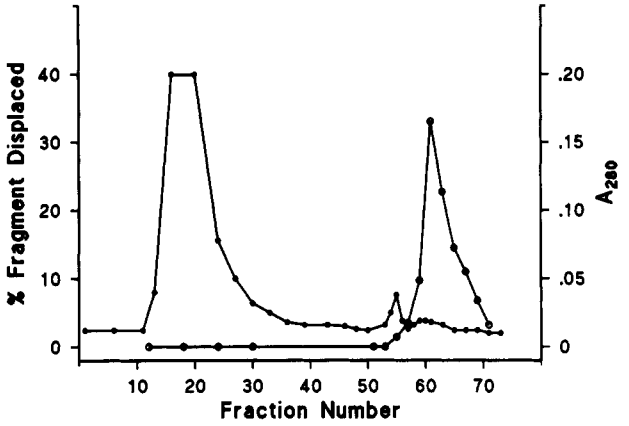

FIGURE 2: Elution profile of δ helicase on single-stranded DNA–agarose. Experimental details are given in the text. Helicase activity was assayed as described in Materials and Methods with the partial duplex substrate containing a 3′-tail (50-mer, 3′-tailed). Incubation was for 60 min at 37 °C. Helicase activity (○) and A_{280} (●) are shown.

helicase activity was recovered in this step and the specific activity of the purified helicase was 180 000 units/mg. The purified helicase was quite unstable and rapidly lost activity on storage at −70 °C.

Physical Properties of δ Helicase. The native molecular size of δ helicase, determined by gel filtration on a Superose-12 FPLC column (Figure 3), is approximately 57 kDa. SDS–polyacrylamide gel electrophoresis of the most active fraction from the single-stranded DNA–agarose column (Figure 4A) revealed two major polypeptides of approximately 52 and 56 kDa, as well as several minor polypeptides. Photoaffinity cross-linking of [α-^{32}P]ATP was carried out to determine which polypeptide(s) binds ATP. As shown in Figure 4B, a broad band of radioactivity was seen centered at approximately 58 kDa, and no other ATP-binding polypeptides were detected.

Enzymatic Characterization of δ Helicase. Figure 5 shows the release of the oligonucleotide from the partial duplex substrate (50-mer, 3′-tailed) as a function of incubation time at the optimal pH (6.5) and $MgCl_2$ concentration (3 mM). The reaction was linear for approximately 30 min and nearly all of the oligonucleotide could be released from the partial duplex.

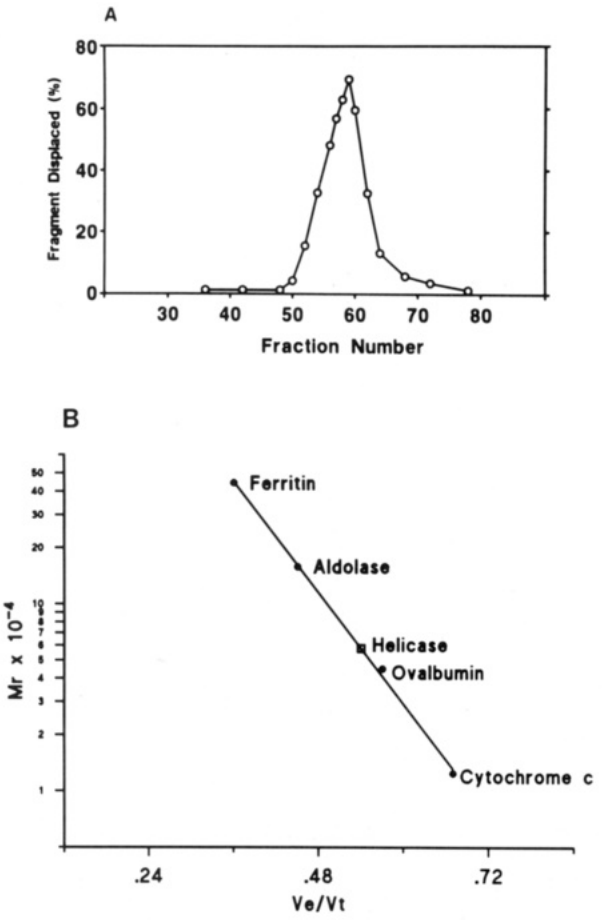

FIGURE 3: Gel filtration of δ helicase. The helicase was chromatographed on a Superose 12 FPLC column (Pharmacia-LKB) in 20 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 30 mM KCl. DNA helicase activity was assayed with the partial duplex substrate containing a 3′-tail (50-mer, 3′-tailed) as described in Materials and Methods. Incubation was for 30 min at 37 °C. (A) Elution profile of δ helicase activity. (B) Molecular weight standards were ferritin (445 000), aldolase (158 000), ovalbumin (45 000), and cytochrome *c* (12 400).

Table I: Nucleotide Requirement for δ Helicase[a]

nucleotide	% fragment displaced	nucleotide	% fragment displaced
none	0	dCTP	1
ATP	62	dTTP	4
GTP	4	ADP	1
CTP	5	AMP	0
UTP	5	AMP-PCP	0
dATP	61	AMP-PNP	0
dGTP	8	ATP-γ-S	1

[a] Helicase activity was assayed as described in Materials and Methods with the partial duplex substrate containing a 3′-tail (50-mer, 3′-tailed). Nucleotides, when present, were at 3 mM. Incubation time was 40 min at 37 °C.

Displacement of the oligonucleotide from the partial duplex requires a nucleoside triphosphate. Table I shows the relative efficiency of several ribo- and deoxyribonucleotides as cofactors in this reaction. Both ATP and dATP are effective cofactors, whereas other ribo- and deoxyribonucleoside triphosphates have little or no activity. AMP and ADP are also ineffective, as are the nonhydrolyzable ATP analogues AMP-PCP and AMP-PNP and the poorly hydrolyzable ATP-γ-S, suggesting that ATP or dATP hydrolysis is necessary for helicase activity.

As is the case with all helicases described thus far, δ helicase also has DNA-dependent ATPase activity. K_m values of 30–40 μM were obtained for both ATP and dATP (data not shown).

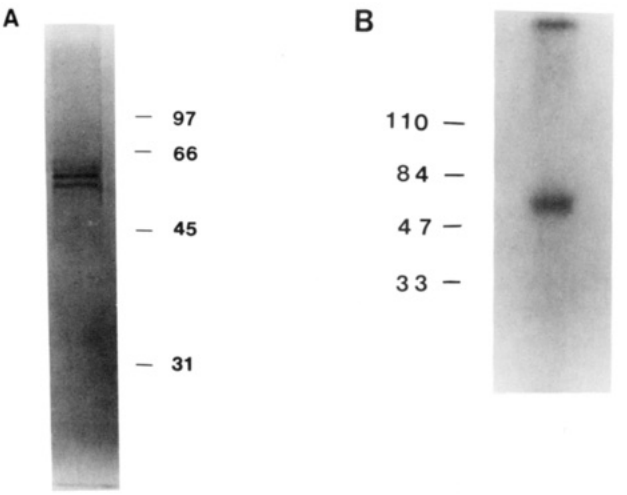

FIGURE 4: Polypeptide composition of δ helicase. (A) The peak fraction from the single-stranded DNA agarose column was resolved on an SDS–10% polyacrylamide gel and silver stained as described in Materials and Methods. Molecular size standards are indicated on the right. (B) The peak fraction from the single-stranded DNA–agarose column was cross-linked with [α-^{32}P]ATP and separated on SDS–PAGE and the [^{32}P]-labeled polypeptide(s) detected by autoradiography as described in Materials and Methods. Molecular size standards are indicated on the left.

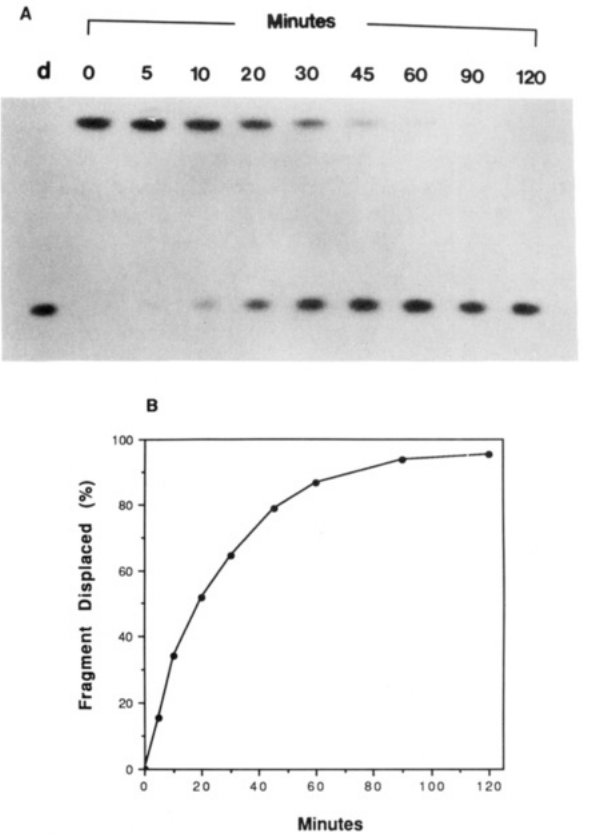

FIGURE 5: Time course of the helicase reaction. Helicase activity was assayed as described in Materials and Methods using a partial duplex with a 3′-tail (50-mer, 3′-tailed). Reaction mixtures contained 6 units of partially purified δ helicase. (A) Autoradiogram of the gel separating the products of the helicase reaction at the indicated incubation times. The lane labeled d contains heat-denatured substrate. (B) Quantitation of the helicase activity in (A).

DNA Binding and Direction of Translocation. In order to investigate the relative affinity of the helicase for single- and double-stranded DNA, polynucleotides of different structures were investigated as competitive inhibitors of the partial duplex substrate (50-mer, 3′-tailed). Figure 6 shows the effects of

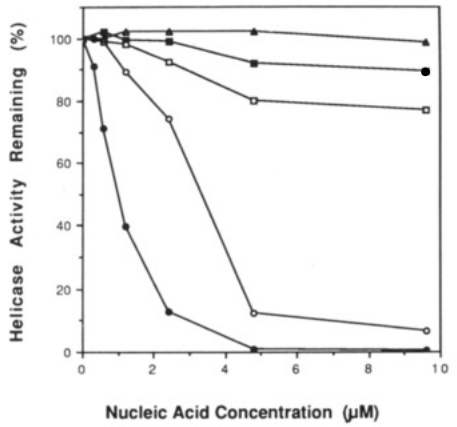

FIGURE 6: Effects of polynucleotides on DNA helicase activity. Helicase assays were carried out as described in Materials and Methods with 4.5 units of partially purified δ helicase. The substrate was the M13 partial duplex containing a 3′-tail (50-mer, 3′-tailed) and incubation time was 45 min. The competitor polynucleotide concentrations were varied as indicated and helicase activity is plotted as a percentage of control activity in the absence of competitor. This corresponds to 62% displacement of the oligonucleotide. M13mp19 (+)-strand DNA (●), poly(dT) (○), poly(dA-dT) (□), pBR322 DNA (■), and poly(A) (△) are shown.

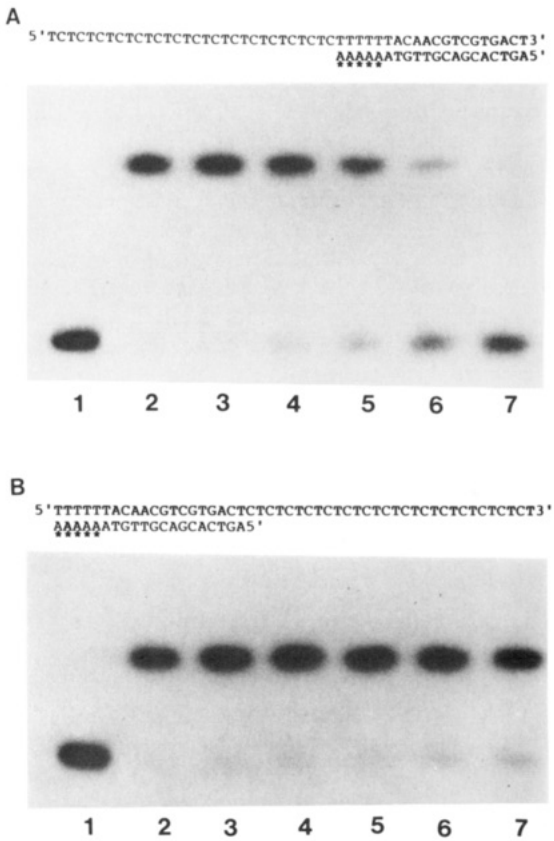

FIGURE 7: Direction of translocation of δ helicase. Helicase assays were carried out as described in Materials and Methods with the substrates whose structures are depicted and single-stranded DNA–agarose-purified δ helicase. Incubation was for 60 min at 37 °C. (A) Substrate for a helicase which translocates 5′ to 3′. (B) Substrate for a helicase which translocates 3′ to 5′. In both panels, lane 1, heat-denatured substrate; lane 2, minus helicase; lane 3, 0.04 unit; lane 4, 0.08 unit; lane 5, 0.16 unit; lane 6, 0.24 unit; and lane 7, 0.48 unit of purified δ helicase.

both single- and double-stranded DNA, as well as several synthetic polynucleotides on helicase activity. The most effective inhibitor was single-stranded M13 DNA, whereas double-stranded pBR322 DNA was not inhibitory, suggesting that the helicase binds to single-stranded DNA. Single-

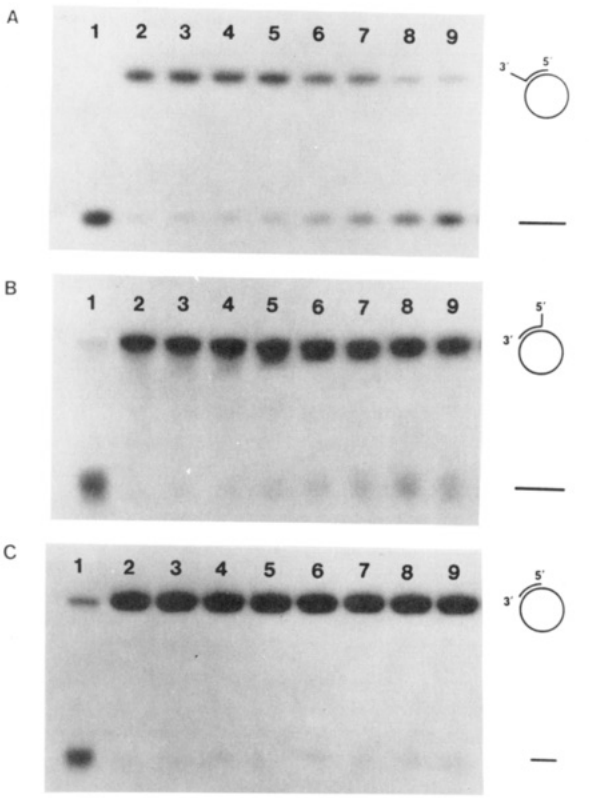

FIGURE 8: Effect of substrate structure on helicase activity. Helicase activity was assayed as described in Materials and Methods with the indicated substrates and amounts of δ helicase. Incubation was for 40 min at 37 °C. (A) The helicase substrate is a partial duplex of M13 DNA containing a 3′-tail on the fragment to be displaced (50-mer, 3′-tailed). (B) The helicase substrate is a partial duplex of M13 DNA containing a 5′-tail on the fragment to be displaced (50-mer, 5′-tailed). (C) The helicase substrate is an untailed partial duplex of M13 DNA (22-mer, untailed). In all panels, lane 1, heat-denatured substrate; lane 2, minus helicase; lane 3, 0.45 unit; lane 4, 0.9 unit; lane 5, 1.8 units; lane 6, 3.6 units; lane 7, 5.4 units; lane 8, 7.2 units; and lane 9, 9 units of partially purified δ helicase.

stranded poly(dT) was inhibitory while single-stranded poly(A) was not, suggesting that the helicase has little or no affinity for RNA.

To investigate the direction of translocation of δ helicase, two partial duplex DNA substrates were prepared. Both substrates contained the same 20-base pair duplex region flanked by a 30-nucleotide single-stranded tail either 5′ to the duplex (Figure 7A) or 3′ to the duplex (Figure 7B). δ helicase preferentially unwound the partial duplex with a 5′-single-stranded tail, suggesting that the helicase translocates in the 5′ to 3′ direction along the strand to which it is bound. A small amount of 20-mer was also displaced from the nonpreferred substrate at high enzyme concentrations. This may be the result of binding of the helicase to a frayed end, rather than translocation in the 3′–5′ direction.

A Preformed Fork Is Required for Maximal Helicase Activity. The effect of substrate structure on the activity of δ helicase was examined by preparing the partial duplex substrates shown in Figure 8. All three substrates have similar lengths and compositions of their duplex regions but they differ in that (A) the 5′-half of the [^{32}P]-labeled oligonucleotide is complemnetary to M13 (+)-strand DNA and the 3′-half is noncomplementary, (B) the 3′-half of the oligonucleotide is complementary to M13 (+)-strand DNA and the 5′-half is noncomplementary, or (C) the oligonucleotide is completely complementary to M13 (+)-strand DNA.

δ helicase is much more efficient in unwinding a partial duplex containing either a 3′- or 5′-tail than a fully comple-

mentary duplex, and a forked substrate with a 3′-single-stranded tail is preferred over a 5′-single-stranded tail. Since the direction of translocation of the δ helicase is 5′ to 3′ on the single strand to which it is bound, it is likely that the helicase unwinds the partial duplex in Figure 8A by translocating along the single-stranded M13 DNA and that in Figure 8B by translocating along the 5′-single-stranded tail.

Discussion

A DNA helicase which differs in physical and enzymatic properties from other eukaryotic DNA helicases described thus far has been purified from fetal calf thymus. The enzyme has an apparent mass of 57 kDa by gel filtration and is associated with polypeptides of 56 and 52 kDa by SDS–PAGE. Photo-cross-linking of the purified enzyme with [α-^{32}P]ATP resulted in labeling of a polypeptide of approximately 58 kDa, suggesting that the active site is present on the larger polypeptide. However, it is not known whether the 52- and 56-kDa polypeptides are related, i.e., whether the 52-kDa polypeptide is a degradation product of the 56-kDa polypeptide. It is also not clear whether δ helicase is the active domain of a larger enzyme, obtained by inadvertent proteolysis during purification.

Unwinding of partial duplex DNA requires a nucleoside triphosphate which may be either ATP or dATP. Other ribo- or deoxyribonucleoside 5′-triphosphates have little or no activity as cofactors, nor do nonhydrolyzable analogues of ATP. Like all known DNA helicases, δ helicase also has DNA-dependent NTPase activity. The K_m value for ATP is approximately 40 μM, which is comparable to that of the yeast *RAD3* helicase (67 μM) (Sung et al., 1987) but considerably lower than those of the calf thymus helicase which partially copurifies with pol α (200 μM) (Thommes & Hubscher, 1990b), mouse ATPase B (750 μM) (Seki et al., 1988), and the *X. laevis* helicase (1 mM) (Poll & Benbow, 1988).

δ helicase binds to single-stranded DNA and has little or no affinity for double-stranded DNA or single-stranded RNA. As is the case for prokaryotic replicative helicases, e.g., the *dnaB* helicase of *E. coli* (LeBowitiz & McMacken, 1986), the gene 41 helicase of phage T4 (Richardson & Nossal, 1988), and the gene 4 helicase of phage T7 (Matson et al., 1983), as well as the HSV-1 helicase–primase (Crute et al., 1988), δ helicase translocates in the 5′ to 3′ direction along the DNA strand to which it is bound. This is opposite to the direction of translocation of SV40 large T antigen (Wiekowski et al., 1988), which is thought to be required to unwind parental strands during the elongation stage of SV40 replication. The direction of translocation of δ helicase is also opposite to that of another calf thymus DNA helicase that partially copurifies with DNA polymerase α (Thommes & Hubscher, 1990b). The pol α-associated DNA helicase also differs from δ helicase in its size, 47 vs 57 kDa, and its ability to use CTP as an energy source. δ helicase appears to be distinct in physical and enzymatic properties from several DNA helicases isolated from human cells. A HeLa cell DNA helicase (RIP100) which is tightly associated with a putative origin-binding protein (Dailey et al., 1990) differs in both size (100 kDa) and direction of translocation (3′ to 5′). Human DNA helicase IV (Tuteja et al., 1991) translocates in the 5′ to 3′ direction but differs from δ helicase in that it does not require a forklike substrate. Helicase IV also differs in size (100 kDa) from δ helicase. Another HeLa cell DNA helicase is a homodimer with a subunit molecular weight of 72000 (Seo et al., 1991). It translocates in the 3′ to 5′ direction and requires a human single-stranded DNA binding protein for activity. Whether any of these mammalian DNA helicases are involved in chromosomal DNA replication awaits further studies.

δ helicase is similar to the prokaryotic replicative helicases in its preference for a forklike DNA substrate with a 3′-single-stranded tail. This type of substrate structure is essential for the helicase activity of *E. coli dnaB* protein and T7 gene 4 protein, as well as for HSV-1 helicase–primase, and is the preferred substrate for T4 gene 41 helicase (Matson & Kaiser-Rogers, 1990). The preference for a forklike substrate may reflect the requirement that a replicative helicase interact with both parental strands at the replication fork.

Unlike the prokaryotic replicative helicases and the HSV-1 helicase–primase, which are closely associated with DNA primase activity, δ helicase has no primase activity; rather, it partially copurifies with DNA polymerase δ, thought to be the leading-strand replicase (So & Downey, 1988, 1992; Blow, 1989; Weinberg et al., 1990; Tsurimoto et al., 1990). DNA polymerase α, which is thought to be the lagging-strand replicase, has a tightly associated DNA primase (Lehman & Kaguni, 1988). A DNA helicase which partially copurifies with pol α translocates in the 3′ to 5′ direction (Thommes & Hubscher, 1990b); i.e., it would open the helix by translocating along the leading-strand template, whereas the pol δ-associated helicase translocates 5′ to 3′, i.e., along the lagging-strand template.

Partially purified pol δ (step 6 enzyme) is capable of carrying out strand displacement synthesis in the presence of PCNA (Downey et al., 1988), whereas the core enzyme has lost this capacity (Downey et al., 1990). The loss of the ability to carry out strand displacement synthesis coincides with the separation of δ helicase from DNA polymerase δ, suggesting that δ helicase may be required for strand displacement synthesis. However, attempts to reconstitute strand displacement synthesis by the combination of purified pol δ core enzyme, PCNA, and purified δ helicase have not been successful, suggesting that one or more factors required for strand displacement synthesis by pol δ may have been removed or inactivated during the purification of the δ helicase.

Although the in vivo function of δ helicase is not known, the present observations that this helicase shares several functional properties with the prokaryotic replicative DNA helicases as well as the replicative helicase–primase of HSV-1, i.e., they translocate in the 5′ to 3′ direction and prefer a forklike DNA substrate, suggest that δ helicase may be a candidate for a eukaryotic replicative helicase.

Acknowledgments

We thank Carlos Castillo for excellent technical assistance in enzyme purification and Xinyi Zhan for help in preparing illustrations.

Registry No. ATP, 56-65-5.

References

Alberts, B. M. (1990) *Nature 346*, 514–515.
Biswas, S. B., & Kornberg, A. (1984) *J. Biol. Chem. 259*, 7990–7993.
Blow, J. (1989) *Trends Genet. 5*, 134–136.
Bruckner, R. C., Crute, J. J., Dodson, M. S., & Lehman, I. R. (1991) *J. Biol. Chem. 266*, 2669–2674.
Crute, J. J., & Lehman, I. R. (1991) *J. Biol. Chem. 266*, 4484–4488.
Crute, J. J., Mocarski, E. S., & Lehman, I. R. (1988) *Nucleic Acids Res. 16*, 6585–6596.
Crute, J. J., Tsurumi, R., Zhu, L., Weller, S. K., Olivo, P. D., Challberg, M. D., Mocarski, E. S., & Lehman, I. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2186–2189.

Daily, L., Caddle, M. S., Heintz, N., & Heintz, N. H. (1990) *Mol. Cell. Biol. 10*, 6225–6235.
Dodson, M. S., & Lehman, I. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1105–1109.
Downey, K. M., Tan, C.-K., Andrews, D. A., Li, X., & So, A. G. (1988) *Cancer Cells 6*, 403–410.
Downey, K. M., Andrews, D. A., Li, X., Castillo, C., Tan, C.-K., & So, A. G. (1990) in *Molecular Mechanisms in DNA Replication and Recombination* (Richardson, C. C., & Lehman, I. R., Eds.) UCLA Symposium on Molecular and Cellular Biology, New Series, Vol. 127, pp 141–152, Alan R. Liss, Inc., New York.
Foiani, M., Lindner, A. J., Hartmann, G. R., Lucchini, G., & Plevani, P. (1989) *J. Biol. Chem. 264*, 2189–2194.
Goetz, G. S., Dean, F. B., Hurwitz, J., & Matson, S. W. (1988) *J. Biol. Chem. 263*, 383–392.
Harosh, I., Naumovski, L., & Friedberg, E. C. (1989) *J. Biol. Chem. 264*, 20532–20539.
Kelly, T. J. (1988) *J. Biol. Chem. 263*, 17889–17892.
Kornberg, A., & Baker, T. A. (1991) *DNA Replication*, 2nd ed., W. H. Freeman, San Francisco, CA.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
LeBowitz, J. H., & McMacken, R. (1986) *J. Biol. Chem. 261*, 4738–4748.
Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., & So, A. G. (1984) *Biochemistry 23*, 1906–1913.
Lehman, I. R., & Kaguni, L. S. (1988) *J. Biol. Chem. 264*, 4265–4269.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Matson, S. W., & Richardson, C. C. (1983) *J. Biol. Chem. 258*, 14009–14016.
Matson, S. W., & Kaiser-Rogers, K. A. (1990) *Annu. Rev. Biochem. 59*, 289–329.
Matson, S., Tabor, S., & Richardson, C. C. (1983) *J. Biol. Chem. 258*, 14017–14024.
McHenry, C. S. (1988) *Annu. Rev. Biochem. 57*, 519–550.
Oh, E. Y., & Grossman, L. (1989) *J. Biol. Chem. 264*, 1336–1343.
Poll, E., & Benbow, R. M. (1988) *Biochemistry 27*, 8701–8706.
Que, B. G., Downey, K. M., & So, A. G. (1978) *Biochemistry 17*, 1603–1606.
Richardson, R. W., & Nossal, N. G. (1989) *J. Biol. Chem. 264*, 4725–4731.
Seki, M., Enomoto, T., Hanaoka, F., & Yamada, M. (1987) *Biochemistry 26*, 2924–2928.
Seki, M., Enamoto, T., Yanagisawa, J., Hanaoka, F., & Ui, M. (1988) *Biochemistry 27*, 1766–1771.
Seo, Y.-S., Lee, S.-H., & Hurwitz, J. (1991) *J. Biol. Chem. 266*, 13161–13170.
So, A. G., & Downey, K. M. (1988) *Biochemistry 27*, 4591–4595.
So, A. G., & Downey, K. M. (1992) *Crit. Rev. Biochem. Mol. Biol. 27*, 129–155.
Stahl, H., & Knippers, R. (1987) *Biochim. Biophys. Acta 910*, 1–10.
Stahl, H., Droge, P., Zentgraf, H., & Knippers, R. (1985) *J. Virol. 54*, 473–482.
Stahl, H., Droge, P., & Knippers, R. (1986) *EMBO J. 5*, 1939–1944.
Stillman, B. (1989) *Annu. Rev. Cell Biol. 5*, 197–246.
Sugino, A., Ryu, B. H., Sugino, T., Naumovski, I., & Friedberg, E. C. (1986) *J. Biol. Chem. 261*, 11744–11750.
Sung, P., Prakash, L., Matson, S., & Prakash, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8951–8955.
Tan, C.-K., Castillo, C., So, A. G., & Downey, K. M. (1986) *J. Biol. Chem. 261*, 12310–12316.
Thommes, P., & Hubscher, U. (1990a) *FEBS Lett. 268*, 325–328.
Thommes, P., & Hubscher, U. (1990b) *J. Biol. Chem. 265*, 14347–14354.
Tsurimoto, T., Melendy, T., & Stillman, B. (1990) *Nature 346*, 534–539.
Tuteja, N., Rahman, K., Tuteja, R., & Falaschi, A. (1991) *Nucleic Acids Res. 19*, 3613–3618.
Weikowski, M., Schwarz, M. W., & Stahl, H. (1988) *J. Biol. Chem. 263*, 436–442.
Weinberg, D. H., Collins, K. L., Simancek, P., Russo, A., Wold, M. S., Virshup, D. M., & Kelly, T. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8692–8696.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, K. R. (1981) *Anal. Biochem. 18*, 197–203.